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Silencing Expression of the Defensin, Varisin, in Male Dermacentor variabilis by RNA Interference Results in Reduced Anaplasma Marginale Infections

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for RNAi were morphologically abnormal from those seen in elution buffer injected control ticks. The colony shape was irregular and in some cases the A. marginale appeared to be free in the cytoplasm of midgut cells. Some ticks were found to be systemically infected with a microbe that may have been related to the silencing of varisin. This appears to be the first report of the silencing of expression of a defensin in ticks by RNAi that resulted in reduced A. marginale infections.

Response to Reviewers: We have revised the manuscript extensively in accordance with the recommendations of the reviewers. In most cases, we accepted the reviewer's suggestions for rewriting the text and substituted those revisions as recommended. There is not sufficient space in this comment box to detail every change that was made to every specific comment, but we did attempt to address all comments from the several reviewers and incorporate them.

Silencing expression of the defensin, varisin, in male *Dermacentor variabilis* by RNA interference results in reduced *Anaplasma marginale* infections

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Abstract

Antimicrobial peptides, including defensins, are components of the innate immune system in ticks that have been shown to provide protection against both gram-negative and gram-positive bacteria. Varisin, one of the defensins identified in *Dermacentor variabilis,* was shown to be produced primarily in hemocytes but transcript levels were also expressed in midguts and other tick cells. In this research, we studied the role of varisin in the immunity of ticks to the gram-negative cattle pathogen, *Anaplasma marginale*. Expression of the varisin gene was silenced by RNA interference (RNAi) in which male ticks were injected with varisin dsRNA and then allowed to feed and acquire *A. marginale* infection on an experimentally-infected calf. Silencing expression of varisin in hemocytes, midguts and salivary glands was confirmed by real time RT-PCR. We expected that silencing of varisin would increase *A. marginale* infections in ticks, but the results demonstrated that bacterial numbers, as determined by an *A. marginale msp4* quantitative PCR, were significantly reduced in the varisin-silenced ticks. Furthermore, colonies of *A. marginale* in ticks used for RNAi were morphologically abnormal from those seen in elution buffer injected control ticks. The colony shape was irregular and in some cases the *A. marginale* appeared to be free in the cytoplasm of midgut cells. Some ticks were found to be systemically infected with a microbe that may have been related to the silencing of varisin. This appears to be the first report of the silencing of expression of a defensin in ticks by RNAi that resulted in reduced *A. marginale* infections.

Keywords: defensin, varisin, RNA interference, *Dermacentor variabilis, Anaplasma marginale.*

Introduction

Ticks transmit a greater variety of pathogens than any other group of hemotophagous arthropods (Sonenshine 1993). In ticks, the midgut is the first site of exposure to a wide variety of hemoparasites that may be ingested with the bloodmeal. Some of these hemoparasites are either not infective for ticks and rapidly digested or cleared by the innate tick immune system. Others infect midgut epithelial cells where they multiply and subsequently infect other tissues including the salivary glands. Transmission may occur when the tick is ingested by the vertebrate host or from salivary glands via the saliva to vertebrate hosts when the ticks feeds again. Tick-borne pathogens have apparently co-evolved with ticks for their mutual survival because, while pathogens undergo considerable multiplication in ticks, these infections do not appear to be detrimental to tick feeding or their biology (Kocan et al. 1992a; Kocan et al. 2005; Sonenshine et al. 2005).

Among the various tick-borne pathogens, those belonging to the genus *Anaplasma* (Rickettsiales: Anaplasmataceae) are obligate intracellular organisms found exclusively within parasitophorous vacuoles in the cytoplasm of both vertebrate and tick host cells (Kocan 1986; Dumler et al., 2001). The type species, *A. marginale*, causes the economically important cattle disease, anaplasmosis, with *Dermacentor variabilis* comprising one of the main tick vectors of this pathogen in the U.S. (Kocan et al., 2004).

While the molecular relationship between ticks and pathogens is not well understood, these molecular interactions may enhance or be necessary for tick and pathogen biology (de la Fuente et al. 2007a). In this emerging area of research, initial studies of tick host cell response to *Anaplasma* infection revealed genes that are differentially expressed in response to pathogen infection. These genes, therefore may be necessary for and facilitate pathogen infection,

multiplication and transmission (i.e. receptors) or limit infections that favor tick survival (de la Fuente et al. 2001; 2005; 2007 a, b; Manzano-Roman et al. 2007).

One component of innate immune systems of eukaryotic organisms are the small cationic peptides known as defensins, which have been identified in a wide range of species ranging from the simplest invertebrates to mammals, as well as plants (Gillespie et al. 1997). Among invertebrates, the most completely characterized defensins contain 6 cysteines and provide immunity against gram-positive bacteria (Ganz and Lehrer 1994; Fogaca et al. 2004). In insects, these defensins were found to be expressed primarily in fat body and midgut epithelial cells (Hoffman and Hetru, 1992; Boulanger et al. 2002).

Defensins have been identified in a variety of ixodid ticks, including *D. variabilis* (Johns et al. 2001a; Ceraul et al. 2003), *Ixodes scapularis* (Hynes et al. 2005), *Amblyomma americanum* (Todd et al. 2007), *A. hebraeum* (Lai et al. 2004) and *R. microplus* (Fogaça et al. 2004; Tsuji et. al. 2007). While defensins have clearly been shown to be expressed in tick hemocytes (Johns et al. 2000; 2001a), they were also found to be expressed or at least transcribed in midguts and other tick tissues in the soft tick *Ornithodoros moubata* (Nakajima et al. 2002) and the hard ticks *Amblyomma americanum* and *Ixodes scapularis* (Todd et al. 2007; Hynes et al. 2005). Tick defensins were shown to be involved in protection against a wide range of organisms such as *Micrococcus luteus* in *Dermacentor variabilis* (Johns et al. 20001a) or *Escherichia coli* and *Staphylococcus aureus* as demonstrated in *A. hebraeum* (Lai et al. 2004). Upregulation of a defensin occurred in response to challenge-exposure of *D. variabilis* with the gram-negative rickettsia, *Rickettsia montanensis,* fed to ticks via capillary tubes (Ceraul et al. 2007). In addition, defensins were also found to provide immunity against the protozoan parasites, *Babesia equi, B. gibsoni* and *B. microti* (Tsuji et al. 2007). This collective

research suggests that defensins contribute to the elimination or modulation of microbes to which ticks are exposed.

In this study we hypothesized that expression of varisin would provide protection in *D. variabilis* against infection by the gram-negative *A. marginale*. RNA interference (RNAi) was used to silence the varisin gene in male *D. variabilis*, after which the ticks were allowed to feed on an *A. marginale*-infected calf to acquire bacteria. Varisin gene silencing was confirmed by real time RT-PCR and *A. marginale* abundance was determined by use of a quantitative PCR assay for *A. marginale msp4* gene. Surprisingly, the results derived from this research were contrary to our hypothesis and demonstrated that silencing of varisin resulted in significantly reduced *A. marginale* numbers. Further studies are needed to determine whether defensin may be necessary for the development of *A. marginale* in ticks.

Materials and Methods

Ticks.

Dermacentor variabilis males were purchased from a laboratory colony maintained at the Oklahoma State University (OSU), Tick Rearing Facility, Stillwater, OK. Larvae and nymphs were fed on rabbits and male ticks derived from the engorged nymphs were used for these studies. Male ticks were used for these studies because they become persistently infected with *A. marginale* and the pathogen's developmental cycle has been well described in the intrastadial cycle. In addition intrastadial studies avoid the possible influence of molting. Off-host ticks were maintained in a 12 hr light:12 hr dark photoperiod at 22-25°C and 95% relative humidity.

Infection of ticks with *A. marginale*.

For infection of ticks with *A. marginale*, male *D. variabilis* ticks injected with either varisin dsRNA or elution buffer alone were allowed to acquire bacteria during feeding

(acquisition feeding, AF). Acquisition was done by feeding the ticks for seven days on a splenectomized calf that was experimentally-infected with the Virginia isolate of *A. marginale* which was shown previously to be infective and transmissible by ticks (Kocan et al. 1992 a, b) when the ascending percent parasitized erythrocytes (PPE) was 3-4%. The ticks were then removed and maintained off-host for 4 days, after which they were allowed to feed for seven days on a sheep to allow for development of *A. marginale* in tick salivary glands and transmission (transmission feeding, TF). Two days after infestation of the sheep all unattached ticks were removed and discarded. All ticks were removed after 7 days of feeding and held in the humidity chamber for four days. The calf and sheep were housed at the OSU Center for Veterinary Health Sciences, Laboratory Animal Resources with a protocol approved by OSU Institutional Animal Care and Use Committee.

RNA interference in ticks.

Oligonucleotide primers homologous to *D. variabilis* defensin and containing T7 promoters for *in vitro* transcription and synthesis of dsRNA (DEFT75: 5′-*TAATACGACTCACTATAGGGTACT*ATGCGCGGACTTTGCATCTGC and DEFT733: 5′-*TAATACGACTCACTATAGGGTACT*TACGTCGACAAAGCGCTTCGG) were synthesized to amplify tick defensin. RT-PCR and dsRNA synthesis reactions were performed as described previously (de la Fuente et al., 2006 a, b), using the Access RT-PCR system (Promega) and the Megascript RNAi kit (Ambion, Austin, TX, USA). The purified dsRNA was quantified by spectrometry (BioRad SMART SPEC 3000).

In order to test the effect of injection with varisin dsRNA on development of *A. marginale* in male *D. variabilis,* 20 ticks per group were injected in the lower right quadrant of the ventral surface of the exoskeleton with approximately 0.4 µl of varisin dsRNA ($5x10^{10}$-$5x10^{11}$ molecules per µl) (de la Fuente et al., 2006a; 2006b). The exoskeleton was first pierced

with the tip of a 30 g needle to create an opening and then the dsRNA was injected through this opening into the hemocoel using a Hamilton® syringe fitted with a 33 g needle. Twenty ticks were injected with *D. variabilis* subolesin dsRNA to serve as positive controls (de la Fuente et al. 2006a, 2006b) or elution buffer used in the final step of purification of dsRNA (10 mM Tris-HCl, pH 7, 1 mM EDTA) alone to serve as negative controls. The ticks were held in a humidity chamber for 24 hr after which they were allowed to feed on an experimentally infected calf.

Analysis of tick attachment and feeding.

Tick attachment was evaluated during AF and TF as the ratio of attached ticks 48 hrs after infestation on the calf to the total number of ticks. Tick mortality was evaluated as the ratio of dead ticks after feeding on the calf (AF) or the sheep (TF) to the total number of fed ticks. Tick attachment and mortality were compared between dsRNA and elution buffer-injected ticks by $\chi 2$–test as implemented in Mstat 4.01 (α=0.01).

Dissection of tick tissues and hemolymph collection for determination of mRNA levels and *A. marginale* infections.

Midguts were dissected from 5 ticks after AF and stored in RNAlater (Ambion) for extraction of DNA and RNA using Tri-Reagent (Sigma) according to manufacturer's instructions to determine the *A. marginale* levels by *msp4* quantitative PCR (de la Fuente et al., 2001) and to confirm gene expression silencing by real-time RT-PCR as described below. After TF, salivary glands and guts were dissected from 5 ticks from each group and processed for RNA and DNA studies as described. Tick tissues were processed and analyzed individually. Midguts and salivary glands were also collected from another 5 ticks and fixed for microscopy studies (see following section).

To assess the effect of defensin RNAi on the expression of defensin in tick hemocytes, 50 male *D. variabilis* ticks were injected with defensin dsRNA or elution buffer alone as

described above. Injected ticks were allowed to feed on a calf for three days after which they were removed with forceps. Hemolymph was collected from the severed legs of two groups of 25 ticks each from both the RNAi and control groups using finely drawn 100 µl glass collecting micropipets (VWR International, Suwanee, GA), and dispensed into 30 µl of sterile phosphate-buffered saline (PBS). Total RNA was extracted and the expression of defensin was quantified by real time RT-PCR as described below.

Real-time reverse transcription (RT)-PCR analysis.

Total RNA was extracted from 5 individual uninfected and *A. marginale*-infected male *D. variabilis* guts and salivary glands and from two hemolymph pools from 25 ticks each using TriReagent (Sigma) according to manufacturer's instructions. Two primers were synthesized based on the sequences of *D. variabilis* defensin (Genbank accession number AY181027; Ceraul et al. 2003) (DvDEFEN-5: TCTGGCATCATCAAGCAGAC and DvDEFEN-3: CTGCAAGTATTCCGGGGTTA) and used for real-time RT-PCR analysis of mRNA levels in uninfected and *A. marginale*-infected ticks. Subolesin mRNA levels were determined as described previously (de la Fuente et al. 2006b). Real-time RT-PCR was done using the QuantiTec SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) and a Bio-Rad iQ5 thermal cycler (Hercules, CA, USA) following manufacturer's recommendations. Amplification efficiencies were normalized against tick β–actin (forward primer: 5′-GAGAAGATGACCCAGATCA; reverse primer: 5′- GTTGCCGATGGTGATCACC) using the comparative Ct method (de la Fuente et al., 2007 a,b). mRNA levels were compared between infected and uninfected ticks by Student's t-Test (P=0.05) and average mRNA levels were used to calculate percent silencing in dsRNA-injected ticks with respect to elution buffer-injected controls.

Quantification of *A. marginale* infections in ticks by PCR.

A. marginale infections in dsRNA injected and control ticks were determined by a major surface protein 4 (*msp4*) quantitative PCR as reported previously (de la Fuente et al. 2001). Total DNA was extracted from 5 individual *A. marginale*-infected and uninfected male *D. variabilis* collected after TF using TriReagent (Sigma) according to manufacturer's instructions. *A. marginale* infection levels in tick midguts and salivary glands were compared between dsRNA and saline injected ticks by Student's t-test (P=0.05).

Light microscopy studies of *D. variabilis* gut and salivary glands.

Ticks were cut in half, separating the right and left halves, and fixed in 2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4). Tick halves were then post-fixed in 0.2 M sodium cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol and embedded in epoxy resin (Kocan et al. 1980). Thick sections (1.0 μm) were cut with an ultramicrotome and stained with Mallory's stain (Richardson et al., 1960). Photomicrographs were recorded using a light microscope equipped with a 3-chip digital camera.

RESULTS

Tick attachment, feeding and *A. marginale* calf infection levels during tick feeding.

Tick attachment and survival after AF (95% attachment and 85% survival) and TF (95% attachment and 89% survival) did not appear to be affected by injection of ticks with varisin dsRNA when compared to the elution buffer (100% and 97% attachment and 88% and 91% survival after AF and TF, respectively; $\alpha>0.01$) and subolesin-injected controls (95% and 100% attachment and 88% and 90% survival after AF and TF, respectively; $\alpha>0.01$). The PPE during tick feeding on the calf experimentally infected with the Virginia isolate of *A. marginale* ranged from 4.8% to 35.9%.

Silencing of expression of varisin in tick tissues.

RNAi resulted in 99.4% silencing of varisin expression in tick hemolymph as determined by real-time RT-PCR (Table 1). Silencing of the varisin gene by RNAi was also confirmed by real time RT-PCR in tick midguts after AF (89%) and in the midguts (97%) and salivary glands (57.9%) after TF as compared with the elution buffer-injected controls (Table 1). For the positive control ticks injected with subolesin dsRNA, silencing in midguts after AF was 90.0%; after TF, it was 99.7% in midguts and 99.4% in salivary glands (Table 1).

The effect of varisin RNAi on *A. marginale* infections in male *D. variabilis*.

Levels of *A. marginale* tick infections, as determined by a *msp4* quantitative PCR and analyzed by Student's t-test, were significantly reduced in tick midguts after AF and in salivary glands after TF as compared with the elution buffer-injected controls (*P≤0.05) (Table 2). Although not statistically significant, *A. marginale* infection levels were also lower in tick midguts after TF as compared with the elution buffer-injected controls (Table 2). Reduction of *A. marginale* levels after RNAi of the subolesin gene (positive control) was statistically significant only in salivary glands collected from transmission fed ticks (Table 2).

Expression levels of varisin in *A. marginale*-infected and uninfected *D. variabilis*

Varisin mRNA levels were higher after TF in the midguts of uninfected ticks as compared to infected ticks (P=0.02). In contrast, varisin levels were significantly higher in the salivary glands from *A. marginale* infected ticks (P=0.05) as compared to the salivary glands from uninfected ticks (Table 3).

Light microscopic changes in ticks injected with varisin dsRNA.

Morphologic changes were observed in the colonies of *A. marginale* in tick midguts after injection of ticks with varisin dsRNA as compared with the elution buffer-injected control ticks. While typical large, round colonies of *A. marginale*, were observed in the control ticks, colonies in the varisin dsRNA injected ticks were irregular in shape (Figs. 1 A and B). Some

tick midgut cells appeared to contain *A. marginale* free in the cytoplasm rather than within the parasitophorous vacuole (Fig. 1B, arrowheads). Hemocytes in the varisin dsRNA injected ticks were degranulated as compared with those from the controls (Figs. 1 C and D). Two of these ticks appeared to be systemically infected with microbes of unknown identity. Large numbers of these organisms were observed in most tissues, including midguts (Fig. 1E) and spermatogonia (Fig 1F). Similar systemic microbial infections were not observed in the elution buffer- or subolesin dsRNA injected controls (data not shown).

Discussion

Ticks are exposed to a wide variety of organisms from mammalian hosts during their extended feeding periods. While some of these organisms are not infective for ticks, others infect tick midguts, where they undergo development and are subsequently transmitted to other hosts during feeding or when the ticks are ingested by the host. During attachment and blood feeding, tick genes express a variety of proteins and peptides involved in the innate immune response that function to inhibit microbial infection, as well as mitigating the oxidative stress and the toxic byproducts (e.g., heme) of hemoglobin digestion. These proteins may include several stress reducing proteins such as glutathione-S-transferases (Dreher-Lesnick et al. 2006), protease inhibitors, lectins and others (Lehane et al. 1997; Zhou et al. 2006, Rudenko et al., 2005). In addition, anti-microbial peptides in ticks have been reported to be upregulated in response to microbial challenge. For example, lysozyme was found to be upregulated in tick hemolymph after challenge-exposure with *E. coli* (Simser et al. 2004).

An example of the ability of ticks to rapidly eliminate noninfective organisms was demonstrated by de la Fuente et al. (2001) in which *D. variabilis* males that fed for 7 days on calves with > 70% erythrocytes infected with a non-tick transmissible isolate (Florida isolate) of

A. marginale were found to be clear of *A. marginale* DNA four days after being removed from the infected calf.

The small cationic peptides, defensins, are a notable part of the innate response in ticks. Defensins were found to be upregulated in response to challenge with *B. burgdorferi* or gram positive bacteria (Johns et al. 2001b; Ceraul et al. 2003; Nakajima et al. 2001, 2002). Upregulation of tick defensins has also been reported in response to gram negative bacteria such as the intracellular rickettsia, *R. montanensis* (Ceraul et al. 2007) and to protozoan pathogens such as *Babesia* species (Tsuji et al. 2007). The reports cited above suggest that ticks are able to eliminate or at least curtail most microbial infections to which they are exposed.

In this research we tested the hypothesis that one of the defensins identified in *D. variabilis*, varisin, was involved in the tick innate immune response in response to infection with the gram negative cattle pathogen, *A. marginale.* If the results supported our hypothesis, silencing the expression of the varisin gene by RNAi would have resulted in greater numbers of *A. marginale* in the ticks. While expression of varisin was confirmed to be silenced in the midguts and hemocytes of the male *D. variabilis* after AF and in the midguts and salivary glands after TF, both sites of varisin expression (Johns et al. 2001a; Ceraul et al. 2003), the results of these studies were opposite to those expected. Silencing of varisin resulted in significantly lower numbers of *A. marginale* organisms in these male ticks. These results suggested that defensin may play a role in *A. marginale* infection and multiplication in *D. variabilis* in a manner different than we had expected. Interestingly, varisin appeared down-regulated in the gut of infected ticks but it was up-regulated in the salivary glands after TF. These results suggest a mechanism by which *A. marginale* may down-regulate varisin expression to establish infection in the guts while in the salivary glands varisin may plays a role in pathogen infection and multiplication.

Although these studies were not designed to quantify morphologic changes, the appearance and integrity of the *A. marginale* colonies in midgut epithelial cells suggested an impact of varisin RNAi on parasite development. Within host cells, *A. marginale* develop within a parasitophorous vacuole (called colonies) which is uniformly round. However, in ticks in which varisin was silenced by RNAi, *A. marginale* colonies were highly irregular and some organisms appeared to be free within the cell cytoplasm.

Another explanation for the reduction in the numbers of *A. marginale* organisms is that it may have resulted from divergent changes in the levels of expression of off-target genes (Scacheri et al. 2004; Ma et al. 2006). At least in mammalian systems, RNAi is known to induce unexpected and divergent changes in the levels of expression of off-target genes (Schaceri et al. 2004). Specifically, in some mammalian systems, RNAi resulted in global upregulation of the interferon system with unexpected consequences (Siedz et al. 2003). Similarly, as reported for *salps* 16 and other tick genes (Sukumaran et al., 2006; de la Fuente et al. 2007c), defensin expression may be manipulated by the pathogen to aid in its multiplication by an as yet undefined mechanism. Alternatively, RNAi treatment may have affected other physiological processes that modified tick susceptibility to infection by other pathogens. Finally, due to the redundant gene function of other defensin genes (Ceraul et al., 2007), the possibility that silencing of the varisin gene targeted in these studies may not be sufficient to suppress all defensin response in ticks should be considered.

Interestingly, other effects were noted in ticks after varisin RNAi. We observed that two of five ticks appeared to have a systemic infection with an unknown microbe. Although the microbes were seen in most tissues, infections were most notable in the midgut and testis. However, similar systemic infections were not seen in sections of five

control ticks (elution buffer- or subolesin dsRNA-injected ticks). While the microscopy studies herein were not designed to be quantitative, this observation provided evidence that the silencing of varisin by RNAi may have been related to extensive multiplication of a microbe other than *A. marginale*. Further studies are needed to define the relationship between other microbes and *A. marginale*. We also noted degranulation of hemocytes in the ticks injected with varisin dsRNA. However, whether either of these observations were directly related to varisin knockdown is not known.

The results reported here illustrate the utility of RNAi as a powerful tool for studying the effect of gene silencing in ticks as reported previously (de la Fuente et al. 2007c). However, the effect of gene silencing may be indirect rather than direct due to off-target RNAi effects and may be limited by our understanding of the molecular biology of tick-pathogen interactions. Since ticks and the pathogens they transmit have co-evolved molecular interactions to assure their survival, these interactions are likely to involve loci in both the pathogen and the tick. Further studies are needed to fully explore the impact of defensins on the infection and development of *A. marginale* in ticks.

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Table 1. Confirmation of gene silencing in midguts, salivary glands and hemolymph from male *D. variabilis* that were injected with varisin and subolesin dsRNA.

Tick tissue/Collection time	Expression silencing ± SD (%)[a]	
	Varisin	Subolesin
Midguts after AF	89.9±0.1*	90.0±21.5*
Midguts after TF	97.4±0.1*	99.7±0.7*
Salivary glands after TF	57.9±0.2*	99.4±0.9*
Hemolymph[b]	99.4±0.5*	ND

[a]Total RNA was extracted from 5 individual ticks from each group and varisin and subolesin expression silencing was determined with respect to control ticks after RNAi. mRNA levels were determined by real-time RT-PCR and compared between dsRNA-treated and control ticks by Student's t-Test (*P<0.05). Amplification efficiencies were normalized against β–actin using the comparative Ct method and average mRNA levels were used to calculate percent silencing in dsRNA-injected ticks with respect to elution buffer-injected controls..

[b]Ticks were allowed to feed for three days after treatment on an uninfected calf and hemolymph was collected from two groups of 25 ticks each. Varisin mRNA levels were determined with respect to control ticks after RNAi by real-time RT-PCR and compared between dsRNA-treated and control ticks by Student's t-Test (*P<0.05) as described above for tick guts and salivary glands. ND, not determined.

Table 2. *Anaplasma marginale* infection levels in *D. variabilis* males that were injected with varisin and subolesin dsRNAs and then allowed to acquire *A. marginale* infection by feeding on an experimentally infected calf.

Tick tissue	Average *A. marginale*/tick ± SD[a]		
	Varisin RNAi	Subolesin RNAi	Control
Midguts after AF	340±535*	814±122	40579±6993
Midguts after TF	1006±470	1517±1025	28252±27788
Salivary glands after TF	2±0*	2±0*	287±144

[a]*A. marginale* infection levels in midguts or salivary glands from 5 ticks per group were determined by *msp4* PCR and compared between dsRNA-treated and control ticks by Student's t-Test (*P<0.05).

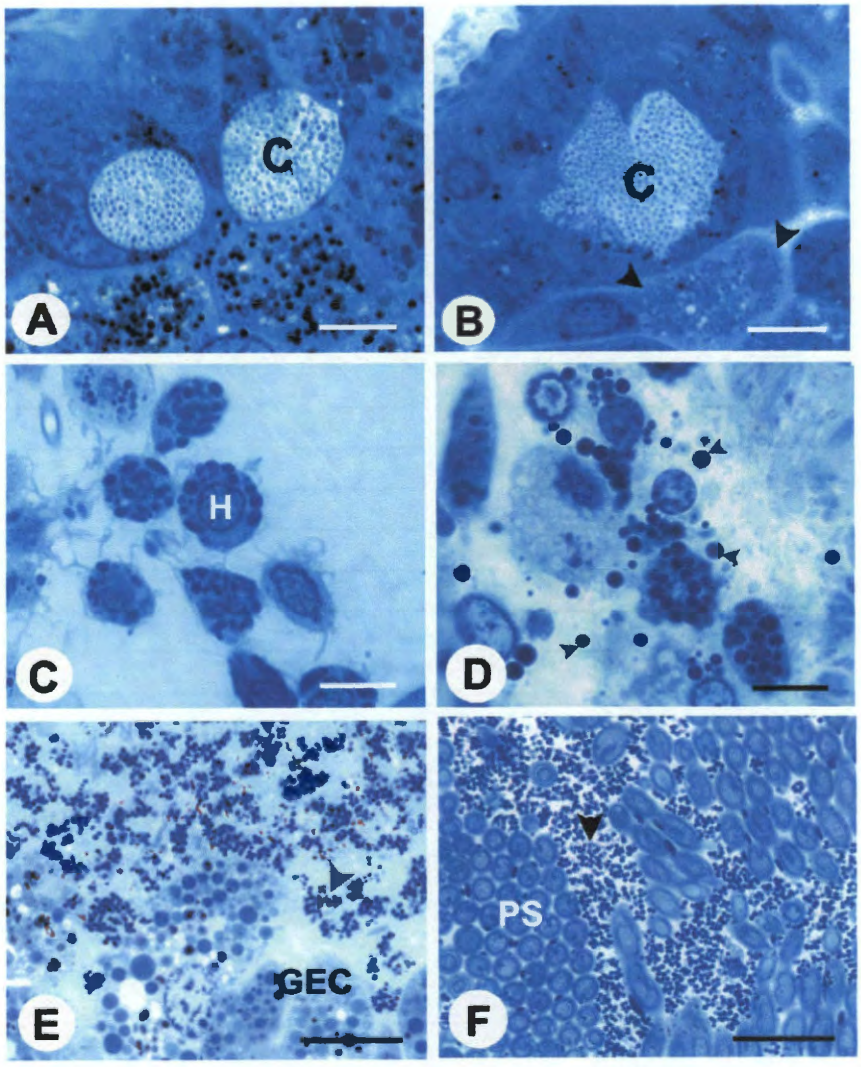
Table 3. Varisin expression levels in *A. marginale*-infected and uninfected *D. variabilis*.[1]

Tick tissue	Average mRNA levels ±SD (arbitrary units)		I/U	P (Student's t-Test)
	Uninfected	Infected		
Gut	5.5±0.6	1.8±1.5	0.3	0.02
Salivary gland	12.5±5.2	29.2±11.1	2.3	0.05

[1]Varisin mRNA levels were determined by real-time RT-PCR and compared between dsRNA-treated and control ticks by Student's t-Test (P=0.05) (N=5). Amplification efficiencies were normalized against β–actin using the comparative Ct method. The infected to uninfected mRNA ratio (I/U) was calculated and showed that defensin mRNA levels significantly decreased in tick guts but increased in tick salivary glands after infection with *A. marginale*.

Figure

Figure 1. Light micrographs of tissues in cross sections of ticks that were either injected with varisin dsRNA or elution buffer to serve as controls. (A) Typical large round colonies (C) of *A. marginale,* as described previously by Kocan et al. (1992a,b), were observed in the midguts of the elution buffer injected control ticks. (B) *A. marginale* colonies (C) observed in the varisin dsRNA males were irregular in shape or appeared to be disrupted in the cytoplasm of gut cells (arrows). (C) Granulated hemocytes (H) were observed in the hemocoel of elution buffer injected control ticks. (D) In contrast to the control ticks, many hemocytes in the varisin dsRNA injected ticks had degranulated (small arrows); (E) Some ticks appeared to be systemically infected with microbes (arrow) which were seen in the midguts lumen (arrow) near gut epithelial cells (GEC) and (F) in spermatogonia (small arrow) among prospermatids (PS). A and B, bars = 10 μm; C and D, bars = 5 μm; E and F, bars = 10 μm.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